Casamino acids slow motility and stimulate surface growth in an extreme oligotroph

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Running head: Casamino acids reduce motility in an oligotroph

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1758-2229.12812

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SUMMARY

Environmental cues that regulate motility are poorly understood, but specific carbon and nitrogen sources, such as casamino acids (CAA), are known to stimulate motility in model organisms. However, natural environments are commonly more nutrient limited than laboratory growth media, and the effect of energy-rich CAA on the motility of oligotrophic microorganisms is unknown. In this study an extreme oligocarbotroph, *Variovorax paradoxus* YC1, was isolated from weathered shale rock within a disused mine level in North Yorkshire, UK. The addition of 0.1 % CAA to minimal media significantly reduced the motility of YC1 after 72 hours, and inhibited swimming motility resulting in enhanced surface growth. We propose this response to CAA is a physiological adaptation to oligotrophy, facilitating the colonization of nutrient rich environments.

INTRODUCTION

Survival in oligotrophic environments requires a suite of physiological adaptations, which often include a reduced cell size and a lack of nutrient specialisation (Hoehler and Jørgensen, 2013). In the case of oligocarbotrophic heterotrophs, organisms that can survive and grow at extremely low levels of organic carbon (< 0.1 mg L^{-1}), the ability to scavenge and utilise trace levels of a range of organic substrates from the surrounding environment is vital (Poindexter, 1981). For example, the ability to catabolise a wider array of amino acids evolves in

populations of *Escherichia coli* maintained in a state of oligotrophy, allowing dead biomass to be more efficiently utilised under nutrient limiting conditions (Zinser and Kolter, 2004).

Such adaptations are important for microorganisms living on rock surfaces, which are often deplete in organic carbon, fixed nitrogen and biologically-available phosphorus (Wainwright et al., 1993; Barton et al., 2007). Photoautotrophs inhabiting rocks exposed to sunlight can provide a source of fixed carbon to the wider microbial community, but environments that are largely isolated from photosynthetic carbon, such as marine sediments (Lever et al., 2015) and caves (Barton et al., 2007; Tebo et al., 2015), are more usually colonized by oligotrophic and chemoautotrophic communities. Previous studies have suggested that the enhanced adhesion of cells to nutrient rich surfaces in marine sediments, and the subsequent establishment of biofilms, are important adaptations for microorganisms that would otherwise be starving in oligotrophic waters (Marshall, 1988).

An adaptation that has received less attention is the capacity to move from areas of low to high nutrient availability, including modes of motility such as swimming or surface swarming. A limited number of studies have investigated the role of motility in oligotrophic, rocky environments. These show that reduced cell size and lower exopolysaccharide (EPS) production in motile bacteria grown under oligotrophic conditions enable cells to penetrate deeper into porous rocks. In the presence of a rich energy source, enhanced EPS production and surface colonisation reduce penetration, as biomass and EPS clog the porous channels within the rock (Jenneman et al., 1985; Lappin-Scott et al., 1988; Lappin-Scott and Costerton, 1990). This transition from swimming in an aqueous environment to surface colonization,

potentially involving swarming motility and/or biofilm formation, is likely an important ecological trait for oligotrophic microbes (Marshall, 1988).

The addition of casamino acids (CAA) to solid medium is known to promote motility in a range of bacterial species including *Pseudomonas aeruginosa* (Köhler et al., 2000; Caiazza et al., 2005), *Salmonella typhimurium* (Harshey and Matsuyama, 1994), *Serratia liquefaciens* (Bees et al., 2002), and *Vibrio* sp. (Kjelleberg et al., 1982). This addition is assumed necessary to meet the high metabolic cost of increased flagellar synthesis associated with motility, particularly swarming (Harshey, 2003). However, most of the strains tested from these species are laboratory model organisms or clinical isolates that are unlikely to have oligotrophic adaptations. The addition of CAA (a rich energy source) to the motility media of an oligocarbotroph may elicit a different response compared to copiotrophs (organisms requiring nutrient rich conditions). In this study, the effect of CAA on the swimming and swarming motility of an extreme oligocarbotroph isolated from a weathered rock surface, *Variovorax paradoxus* YC1, was investigated.

RESULTS AND DISCUSSION

Isolation of *Variovorax paradoxus* YC1 from a weathered shale environment

Samples of weathered rock from a 17th century mine level previously sampled (Cockell et al., 2011) were taken to enrich for oligocarbotrophic organisms. Rock samples were used to inoculate enrichment cultures in a minimal medium (M9) lacking an added carbon source. A serial culture transfer series was then propagated in M9 and then milli-Q water over a 7 ½ month period, until the original enrichment culture was diluted by 10^{33}-fold after numerous

rounds of dilution. The surviving microbial community, deemed likely to be enriched in extreme oligocarbotrophs, was plated onto nutrient agar and cultured bacterial isolates identified via phylogenetic analysis. From these isolates, *Variovorax paradoxus* YC1 was chosen for further physiological analysis, based upon the known swimming and swarming capacity of this species (Jamieson et al., 2009; Pehl et al., 2012). A more detailed description of the isolation of *V. paradoxus* YC1 is provided in the supplementary methods.

Demonstration of oligotrophic growth

The purpose of this study was to investigate the effect of a commonly used motility stimulant, casamino acids, on a motile oligotrophic bacterium. Growth in our enrichment culture series containing a community of oligotrophic microbes was sustained without the addition of an added carbon source. Therefore after isolation of *Variovorax paradoxus* YC1, we aimed to demonstrate its ability to grow under these conditions in axenic culture. Growth experiments were conducted with YC1 in M9 medium with and without an added carbon source, with effort taken to reduce contaminating organic carbon in the experimental setup (see supplementary methods). The addition of an added carbon source increased the YC1 population carrying capacity (9×10^6 CFU mL^{-1} compared to 3×10^5 CFU mL^{-1}) (Figure 1) and a two-way ANOVA test performed in R (version 3.5.3) (Team, 2019), with incubation time and media type as predictor variables and log CFU mL^{-1} as the response variable, demonstrated this effect to be significant ($F(1, 23)=27.66$, $p=2.46 \times 10^{-5}$). However, growth in the M9 medium without an added carbon source was still substantial, being over three orders of magnitude higher than the starting density (5×10^2 CFU mL^{-1}) (Figure 1). This indicates that despite extensive effort to eliminate the growth of YC1 by reducing the levels

of contaminating or biologically stored carbon in our culturing approach, YC1 still maintained high levels of growth (~10^5 CFU mL^{-1}) in the absence of an added carbon source.

Potential sources of organic carbon that sustained oligotrophic growth

Growth in the M9 cultures were likely supported by an organic carbon source that was unaccounted for in our experimental design, however the exact nature of this source could not be verified. Trace levels of organic carbon from the inoculum used to initiate the experiment potentially supported some of the observed growth. The most significant carbon source from this inoculum, glycerol (0.25 μg L^{-1}), would only have provided 2.5 ng of organic carbon to a 10 mL culture. Based upon the average carbon content of an *E. coli* cell (35 pg) (Makarieva et al., 2008), which are similarly sized to *V. paradoxus* (Willems et al., 1991), this amount of carbon could not have supported our observed levels of growth (~3 x 10^6 cells). The production, storage and subsequent utilisation of polyhydroxyalkanoates (PHAs) under nutrient limiting conditions is a common mechanism used by oligocarbotrophs for survival. One strain of *V. paradoxus* has been shown to store PHAs (Maskow and Babel, 2001), which provides a potential explanation for our results if YC1 also has this capability.

Geller (Geller, 1983) found that populations of *Pseudomonas fluorescens* could be cultivated in minimal medium lacking an added carbon source, due to the influx of airborne organic substances from laboratory air that dissolved into liquid cultures. The study calculated that this influx increased the dissolved organic carbon concentration by up to 0.5 mg L^{-1} per week, facilitating substantial growth (Geller, 1983). A similar effect could explain the results obtained in this study, with particulate organic material providing the majority of the carbon used by the YC1 populations in M9 media.

Although we cannot provide an exact mechanism by which *V. paradoxus* YC1 scavenges sufficient organic carbon to support such large population sizes, our findings do demonstrate that this organism is an extreme oligocarbotroph, capable of growth in media containing <0.1 mg mL^{-1} organic carbon. As such, YC1 is a suitable candidate to explore the effect of nutrient rich environments (e.g. the presence of casamino acids) on the motility of oligotrophic organisms.

Casamino acids reduce motile colony size, promote surface growth and induce a swarming phenotype

To determine the effect of casamino acid supplement on the motility of YC1, colonies were grown on plates with differing concentrations of agarose, with and without 0.1 % (w/v) CAA. This concentration of CAA was chosen based upon its use in the singular study that has investigated the effect of differing carbon and nitrogen sources on the motility of *Variovorax paradoxus* (Jamieson et al., 2009), enabling a comparison of our results with this previous study. However, similar concentrations ranging from 0.05 to 0.5 % have been used in other investigations with bacterial species including *S. typhimurium*, *P. aeruginosa* and *S. liquefaciens* (Harshey and Matsuyama, 1994; Köhler et al., 2000; Bees et al., 2002; Caiazza et al., 2005). Increasing agarose concentration in the absence of CAA reduced colony size from 20-25 mm at 0.3 % agarose to 5 mm at 1 % agarose (Figure 2). Visual assessment of the colonies revealed that at 0.3-0.5 % agarose cells are swimming through the agar, where no visible growth can be seen on the agarose surface, and a central clear zone in the plate can be seen where cells have migrated from the point of inoculation (Figure 3, bottom left). Above 0.5 % agarose growth is visibly at the plate surface, and a layer of secreted wetting agent

(Jamieson et al., 2009) can be seen beyond the colony edge. This suggests that at 0.3-0.5 % agarose, the pore size is large enough to enable YC1 cells to swim through the gel, whereas above 0.5 % reduced pore size prevents swimming and cells grow and potentially swarm (as indicated by the presence of wetting agents) on the gel surface (Figure 3, top left).

However, in the presence of 0.1 % CAA, colony diameter after 72 hours was reduced to ~5 mm at all agarose concentrations (Figure 2). Furthermore, swimming cells were no longer observed at lower agarose concentrations (0.3-0.5 %), with all growth being found at the agarose surface (Figure 3, top right). A one-way ANCOVA revealed that the effect of agarose concentration (predictor variable) on colony diameter (response variable) was significantly altered by the addition of CAA (covariate), $F(2, 44) = 155.67$, $p=4.76 \times 10^{-16}$.

After 6 days of growth, colonies on plates containing below 0.8 % agarose and 0.1 % CAA had formed complex dendritic patterns of growth (Figure 3, bottom right) that are characteristic of swarming motility (Kearns, 2010). These dendritic colony morphologies were not found on agarose plates lacking CAA, suggesting the addition of CAA did induce swarming motility, even if it also reduced the extent of colony growth and motility. Previous studies have supplemented motility media with CAA to stimulate both swimming and swarming motility (Harshey and Matsuyama, 1994; Bees et al., 2002; Caiazza et al., 2005), indicating that CAA did not induce surface growth in these organisms as it did for *V. paradoxus* YC1. Finally, this entire motility experiment was duplicated, but with agar as the gelling agent instead of agarose, with the effect of CAA on *V. paradoxus* YC1 motility being verified (Supplementary Figure 1).

Jamieson et al. (Jamieson et al., 2009) investigated the effect of carbon and nitrogen source on the motility of *V. paradoxus* strain EPS, isolated from the rhizosphere community of a sunflower plant (*Helianthus annuus*) (Han et al., 2013). They show that on M9 medium supplemented with CAA (0.1 % w/v) as a sole carbon and nitrogen source, EPS swarming colonies were larger (~25 mm at 24 hours) than colonies grown on media containing glucose and ammonium chloride (5-10 mm) (Jamieson et al., 2009). These results are in contrast to those found in this study, where the addition of 0.1 % CAA significantly reduced colony diameter after 72 hours of growth and motility (Figures 2 and 3). These results also contrast with numerous studies published on bacterial swarming motility in other species, where the addition of CAA is assumed to enhance the extent of swarming motility (Kjelleberg et al., 1982; Harshey and Matsuyama, 1994; Köhler et al., 2000; Bees et al., 2002; Harshey, 2003; Caiazza et al., 2005). In support of our findings, Kjellberg et al. (Kjelleberg et al., 1982) found that the growth and motility stimulatory effects of CAA on *Vibrio* sp. DW1 only occurred in the presence of a surface.

Combined results are better explained by a specific physiological response, rather than a toxicity response or phenotypic decay

A potential explanation for the slowed colony growth is that addition of amino acids induced a toxicity response that slowed growth. The addition of single amino acids such as cysteine and threonine to media at low concentrations (1mM, ~0.01 %) inhibits growth in some obligate oligocarbotrophs (Sato et al., 1993). The mechanism behind this growth inhibition is unclear, but the addition of a mixture of amino acids, rather than one single type, is known to counteract this growth inhibition (Ingram and Jensen, 1973). We believe our observed

physiological response to CAA is a specific response, as opposed to toxicity-induced growth inhibition, for two reasons. Firstly, the addition of CAA had three main effects on YC1 motility a) slowed colony growth and motility (Figure 2), b) induced surface growth at low agarose concentrations and c) dendritic swarm morphology (Figure 3). None of these effects were observed in the absence of CAA. This combination of phenotypic changes indicates a specific physiological response to CAA, rather than a general stress response. Secondly, YC1 can be cultured on nutrient agar, with colonies growing to a few millimetres in size after 1-2 days. Nutrient agar contains high concentrations of protein and amino acid sources, such as yeast extract (0.2 %), peptone (0.5 %) and lab-lemco powder (0.1 %) (Lapage et al., 1970). It therefore seems unlikely that YC1 could be cultivated on this media, if a mixture of amino acids induced a toxicity response.

Another alternative interpretation of our results could be based upon the state of motile phenotypes in YC1, which could have been in the process of decay under relaxed selection during growth in the enrichment transfer series. In the absence of a selective pressure for a phenotypic trait, particularly one as metabolically expensive as motility (Harshey, 2003), genetic drift or selection against that phenotype can result in the loss of the unused trait (Hall and Colegrave, 2008). This concept is particularly applicable to oligotrophs, which in nutrient-deplete environments are under constant selective pressure to be metabolically efficient (Roller and Schmidt, 2015). As such, reduced motility in the presence of CAA could be the product of motile phenotypes in YC1 undergoing decay due to the relaxed selective conditions of laboratory cultivation. However, our results demonstrate that the addition of CAA completely inhibited an otherwise strong swimming motility phenotype (Figure 3,

bottom left) and promoted swarming morphology (Figure 3, bottom right), although at a reduced rate of colony expansion. These results do not seem to support the loss of motile phenotypes in YC1. Flagellar machinery synthesis and flagellar activity are both known to be large metabolic costs in their own right, accounting for ~2 % total metabolic expenditure in motile bacteria (Martínez-García et al., 2014). It therefore seems unlikely that the swarming motility phenotype would be under decay if phenotypes relating to flagellar synthesis and activity were not.

After consideration of alternative hypotheses, we propose that the results presented in this study can be best explained by a specific physiological response to CAA in YC1. Reducing motility and inducing surface growth upon exposure to CAA, or other energy-rich substrates, would likely be advantageous to oligotrophic microbes that encounter nutrient-replete surfaces.

Conclusions and future research

The results of this study suggest that the physiological response of *V. paradoxus* YC1 to casamino acids is an adaptation to the environment it was isolated from, as this response differs markedly from that of another strain of *V. paradoxus* (Jamieson et al., 2009) and other motile organisms (Kjelleberg et al., 1982; Harshey and Matsuyama, 1994; Köhler et al., 2000; Bees et al., 2002; Harshey, 2003; Caiazza et al., 2005). We propose that this response is an adaptation to oligotrophic, rocky environments, where reduced motility and surface growth in nutrient rich conditions is advantageous (Marshall, 1988; Lappin-Scott and Costerton, 1990). When a motile oligotrophic microorganism encounters nutrient rich

conditions, reduced levels of motility and transitioning from liquid-based (swimming) to surface-based motility (swarming) likely facilitates colonization of that environment.

Future research should further explore the concepts presented in this publication by investigating the effect of casamino acids, along with other energy-rich substrates, on the motility of bacteria isolated from both copiotropic and oligotrophic environments. We predict that the response of *V. paradoxus* YC1 to casamino acids is not unique to this strain, but is likely present in other motile oligotrophs. In contrast, casamino acids are less likely to have this effect in copiotrophic bacteria (including many motile model organisms studied previously) which are less adapted to energy limiting environments.

ACKNOWLEDGEMENTS

We would like to thank the anonymous reviewers and the editor for their guidance in preparing this manuscript for submission and publication.

DISCLOSURE STATEMENT

ICL Boulby operate an active mine within the local area of the field site sampled in this study.

FUNDING

ICL Boulby provided a PhD studentship for T.S.

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FIGURE LEGENDS

FIGURE 1. Demonstration of oligotrophic growth in *Variovorax paradoxus* YC1 in the absence of an added carbon source. Growth curves (CFU mL^{-1}) in M9 medium either with (black) or without (grey) an added glucose (0.4%) are presented. Biological replicates (N=3)

were used for both conditions. Error bars represent standard error of the mean average. Figure produced in the package ggplot2 in R (Wickham, 2016). In order to starve cells and deplete stored intracellular carbon (e.g. PHA), initial cultures were inoculated from a frozen glycerol stock into 10 mL TOC-free water (1:1000 dilution). This culture was cultivated for three days at 30 ^{o}C, before being further diluted into fresh TOC-free water (1:1000 dilution) and allowed to grow for a further three days. This second dilution in water was used to inoculate (1:1000 dilution) triplicate 10 mL cultures of M9 medium with or without an added carbon source (glucose, 0.4 % w/v). Trace amounts of glycerol (0.25 μg L^{-1}) and glucose (0.002 μg L^{-1}) were transferred to these M9 cultures, derived from the diluted glycerol stock inoculum. Furthermore, carbon could have been supplied through dead biomass transferred from the previous water culture, or from intracellular stores of PHA within the cells inoculated. Growth (CFU mL^{-1}) in each culture was monitored by plating out serial dilutions of each culture onto nutrient agar at 24 hour intervals for four days.

FIGURE 2. Colony diameter of *V. paradoxus* YC1 swarming and swimming colonies after 72 hours of growth on M9+glucose agarose plates at varying agarose concentrations (0.3-1.0 %), with (black) and without (grey) 0.1 % CAA. Biological replicates (N=3) were used for both conditions. Error bars represent standard error of the mean average, but are hidden at some points due to low SE values. Figure produced in the package ggplot2 in R (Wickham, 2016). Triplicate plates were prepared with differing concentrations of agarose (0.3–1.0 % w/v, in 0.1 % increments), with and without 0.1 % (w/v) CAA. Pore size within the gel decreases with increasing agarose concentration , meaning that cells will be able to swim through lower concentration agarose gels (~0.3-0.5 %), but will be forced to grow on the

surface of agarose gels at higher concentrations (~0.6-1.0 %) (Bees et al., 2002). From a frozen glycerol stock, a liquid culture of M9-glucose (0.4 % w/v glucose) was inoculated (1:1000 dilution) and incubated overnight at 30 ºC. This liquid culture was used to inoculate single 5 µL spots onto the centre of individual agarose plates, which were then incubated at 30 ºC for three days. Triplicate measurements of colony diameters (mm) were taken using a standard ruler under a dissection microscope. A distinction was made between the colony edge and the surfactant edge, as previously described (Jamieson et al., 2009). Plates were incubated for a further three days, and photographs taken of representative colonies at the end of this six-day period. Colony diameters were not measured again after 6 days, as many colonies had grown to the plate edge and therefore further colony growth was obstructed.

FIGURE 3. Images of *V. paradoxus* YC1 colonies after 6 days of growth. Top left, 0.7 % agarose plates, insert is a smaller copy of the same image but with the colours inverted to make the colony outline clearer; Top right, 0.7% agarose plates amended with CAA (0.1 %); Bottom left, 0.4 % agarose plates; Bottom right, 0.6 % agarose plates amended with CAA (0.1 %).